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TITLE: A Novel Field-Deployable Point-of-Care Diagnostic Test for Cutaneous Leishmaniasis

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14. ABSTRACT **Leishmaniasis is caused by the protozoan Leishmania and is generally transmitted by the bite of sand flies of the genus Lutzomyia or Phlebotomus, The disease has significant global impact, producing 10-20 million cases of leishmaniasis worldwide. Cutaneous leishmaniasis (CL) is characterized by chronic skin ulcers that can impact the individual's functional status, lead to expensive and untimely treatment, and result in disfiguring scarring. Military training and combat operations resulted in cases of CL in soldiers (USA, UK) deployed to Central America. More recently (2003-2004), CL was reported in almost 1,200 members of the U.S. Armed Forces deployed to Iraq and Afghanistan, and the infection is an ongoing concern in the OEF/OIF veteran population. To date, there is no field-standardized molecular method based on sensitive DNA amplification coupled with Lateral Flow reading to detect leishmaniasis. Isothermal amplification by RPA (Recombinase Polymerase Amplification) is a novel strategy to diagnose infectious diseases that can be used at the POC because it is highly sensitive, fast, inexpensive and able to work at most ambient temperatures.**

15. SUBJECT TERMS

Nothing listed

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Leishmaniasis is caused by the protozoan *Leishmania* and is generally transmitted by the bite of sand flies of the genus *Lutzomyia* or *Phlebotomus,* The disease has significant global impact, producing 10-20 million cases of leishmaniasis worldwide. Cutaneous leishmaniasis (CL) is characterized by chronic skin ulcers that can impact the individual's functional status, lead to expensive and untimely treatment, and result in disfiguring scarring. Military training and combat operations resulted in cases of CL in soldiers (USA, UK) deployed to Central America. More recently (2003-2004), CL was reported in almost 1,200 members of the U.S. Armed Forces deployed to Iraq and Afghanistan, and the infection is an ongoing concern in the OEF/OIF veteran population. To date, there is no field-standardized molecular method based on sensitive DNA amplification coupled with Lateral Flow reading to detect leishmaniasis. Isothermal amplification by RPA (Recombinase Polymerase Amplification) is a novel strategy to diagnose infectious diseases that can be used at the POC because it is highly sensitive, fast, inexpensive and able to work at most ambient temperatures.

1. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Cutaneous leishmaniasis-diagnosis-point of care-DNA amplification-field applicable-isothermal amplification-protozoan parasite

2. ACCOMPLISHMENTS

Specific Aim	Month	% completion
Aim 1: To use simulated field conditions to optimize and produce the established RPA lateral flow diagnostic test for POC deployment.		
Sub-Aim 1.2: To determine if a simple DNA extraction method will provide adequate sensitivity for optimal test function under field conditions. Comparison of DNA yield, sufficient for RPA-LF test using a DNA mini-extractor vs. Whatman FTA filter paper utilizing dermal tissues spiked with *Leishmania* grown in the lab	1-3	100% Lab assays were completed. Clinical samples from the field (NAMRU-6) have been evaluated using a simple extraction method. Results were satisfactory
Sub-Aim 1.3: To determine if subgenus- and/or species-specific primer-probe sets can achieve the same analytical sensitivity and specificity as the genus specific primer-probe set using *Leishmania* isolates and clinical specimens from the field sites.	3-12	100% The analytical sensitivity of the RPA-LF was established for *Leishmania Viannia* spp., *L. major* and *L. enriettii*
Kickoff Coordination Meeting of participating institutions	3	100% A UTMB meeting was organized with participants of all three study sites
Protocol submission for local IRB approval and HRPO approval	3	N-6 100% N-3 90%. RSB renewal in progress; IRB NMRC pending final approval
Implementation of molecular laboratory in Madre de Dios and technology transfer of kDNA PCR procedures from Lima to Madre de Dios for on-site Leishmaniasis diagnosis in the endemic area	6-12	100% Training was completed and equipment purchased. Lab construction was completed, and patient samples are obtained on a regular basis. Still no RPA-LF test has been performed on site.
Milestone Achieved: Local IRB and HRPO approved protocols	6	UTMB 100% NAMRU-6 100% NAMRU-3 80%
<u>Aim 2</u>: To prospectively determine the diagnostic sensitivity and specificity of the RPA-lateral flow test for diagnosis of cutaneous leishmaniasis.		
Sub-aim 2.1. To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RAP-Lateral Flow vs. standard kDNA PCR at NAMRU-6; Lima and	12-48	NAMRU-6 60% 135/222 clinical samples from

Puerto Maldonado, Madre de Dios, Peru Delivery of subset of positive and negative clinical samples (10%) from NAMRU-6 to UTMB for reproducibility testing		patients suspicious of having cutaneous leishmaniasis were obtained in Madre de Dios, Peru. PCR was carried out at NAMRU-6, Lima.
Technical meeting at NAMRU-3, Ghana	14	Not accomplished. However, Naiki Puplampu attended an RPA-LF workshop at N-6 and is fully trained to run the test in Ghana
Sub-aim 2.2. To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RPA-Lateral Flow vs. standard PCR at NAMRU-3, Ghana detachment, Noguchi Memorial Institute for Medical Research, Ho Volta region Delivery of subset of positive and negative clinical samples (10%) from NAMRU-3 to UTMB for reproducibility testing	12-48	As mentioned in aim 2.1 the work has reached 60% completion in the NAMRU-6-UTMB component. NAMRU-3 activities have been delayed due to multiple rounds of IRB questions that required resubmissions. Approval is still pending.
Technical meeting at NAMRU-6, Peru	24	100% A Coordination meeting at NAMRU-6, Peru was carried out in May 2016

Milestone(s) Achieved:

- All the activities between NAMRU-6 and UTMB are in course and the percentage completion of the aims are adequate since a one-year extension of the project was granted.

- The workshop that focused on the utilization of RPA-LF diagnostic test (already reported in the past annual report) has provided researchers of both NAMRUs the necessary experience to confidently process clinical samples at both sites.

- As of October 31, 2017, 135 clinical samples from patients suspicious of having cutaneous leishmaniasis were obtained in Madre de Dios and processed by PCR and RPA-LF at NAMRU-6, Lima.

- We have previously reported that the evaluation of 85 clinical samples carried out during the first phase of the study showed that the RPA-LF test had a sensitivity of 95.06% while the laboratory-based PCR used as gold standard had a sensitivity of 96.29%. Now the sensitivity has been slightly increased reaching a level very similar to PCR.

- Additional work in UTMB during 2017 showed that increasing the proportion of primers in the test resulted in improved sensitivity for detecting *Leishmania* and other pathogens as well. Based on these results, we reevaluated a small number

of samples (within the former 85 samples) that were positive by PCR but resulted negative by RPA-LF (**Figure**). Sample #2 was initially negative by all tests and was included as an additional negative control. The utilization of the newly optimized RPA-LF protocol using the DNA of putatively negative samples stored at -20ºC indicated that 2 more samples were positive for *Leishmania Viannia sp.* (#44 and #48) (**Figure**, upper panel). A second approach with the samples that tested negative was to extract their DNA from samples still preserved in the filter papers (>one year). With this method an additional patient was positive (#24) (**Figure**, lower panel).

These experiments confirmed the importance of running the test from recently extracted DNA; in this case from FTA filter papers rather than DNA stored at -20ºC. RPA-LF is more affected than PCR when the amplification is carried out using DNA that has been repeatedly frozen and thawed. However, this should not be of major concern since the test is meant to be used in the field with freshly obtained samples. When this is not possible, the preservation and transport of samples preserved in filter paper will also be adequate for running RPA-LF.

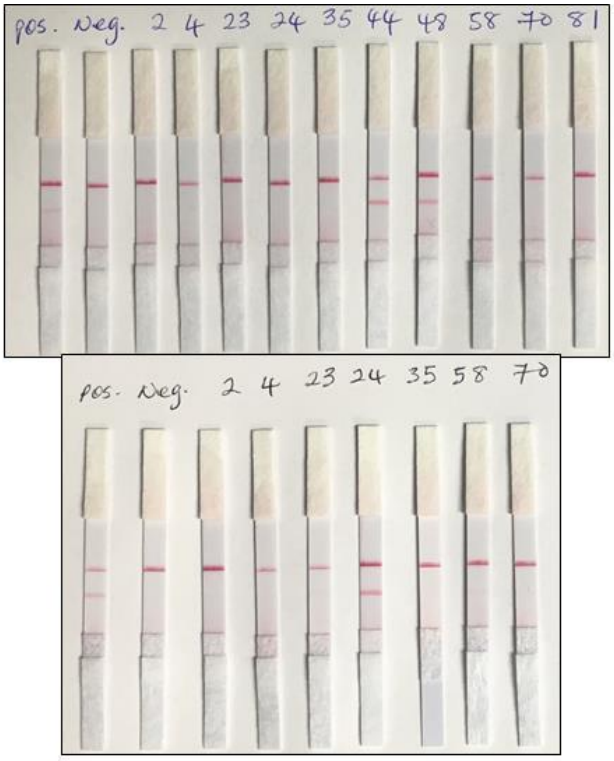

Figure. The upper panel shows samples that were negative by standard RPA-LF. The DNA sample stored at -20ºC was re-tested using a higher concentration of primers. With this protocol two more clinical samples showed positive results. The lower panel depicts results obtained after the negative samples of the upper panel were re-tested after a new extraction of DNA from filter paper was used for immediate RPA-LF testing. With this approach a third sample became positive.

Training Activities

Two laboratory persons were trained in UTMB and are currently working with this POC test. Thomas Shelite PhD, has replaced Omar Saldarriaga PhD and is in charge of processing any samples delivered to UTMB from NAMRU 6 or NAMRU 3. Additionally, Aseruchi Chindah BSc has joined the UTMB lab and is currently involved in the reprocessing of discordant samples. Both persons are fully capable of assisting local or foreign collaborators in the training of RPA-LF utilization for Leishmania or other pathogens.

Results disseminated to communities

Nothing to report

Plans for the next reporting period

We expect that NAMRU-3 will obtain IRB approval to initiate their corresponding field work.

The collection of clinical samples at NAMRU-6 (Pto. Maldonado) and processing in Lima is on schedule.

The one-year extension of the project will allow evaluating the applicability of RPA-LF in the basic lab setting of Madre de Dios, Peru. Likewise, we expect that N-3 will be collecting patient samples and evaluating the feasibility of applying RPA-LF in the Ho endemic region of Ghana. These activities will establish which logistical aspects surrounding RPA-LF implementation have to be considering for the successful utilization of this POC test.

4. IMPACT

Impact on the development of the principal discipline(s) of the project:

The impact remains the same, as reported be fore. The RPA-LF is an innovative diagnostic test to detect cutaneous leishmaniasis, which affects populations in tropical or subtropical countries. Its sensitivity and specificity and potential field applicability would improve diagnosis in civilians and also military personnel deployed in endemic countries.

Impact on other disciplines:

This diagnostic method, which is based on the isothermal amplification of DNA, is impacting the field of molecular biology. It is expanding the concept of instrument-free diagnosis of infectious disease and amplification of DNA for multiple purposes in biology

and medicine.

Impact on technology transfer:

Once the RPA-LF has been validated in the field it will likely be transferred to a commercial company and subsequently make available to the public.

Impact on society:

The development of this diagnostic method, which is sensitive and requires minimal training, will improve the quality of life of populations living in endemic areas. The availability of RPA-LF in economically depressed regions will improve the diagnostic capacity. This will lead to early treatment which will significantly decrease the negative impact of disease.

5. CHANGES/PROBLEMS

The IRB approval for NAMRU-3 (Ghana Detachment) is seriously delayed. It is still in the review stage which started in August 2016. This is preventing the initiation of the field work aimed at collecting patient samples and evaluating the implementation of the RPA-LF in the Ho endemic region.

The implementation of RPA-LF in Puerto Maldonado has been delayed but will take place in the coming months. This will allow assessing the efficacy of RPA-LF in laboratories with basic infrastructure such as that of Puerto Maldonado. Similarly, we expect that a similar evaluation will be carried out by NAMRU-3 in Ghana.

Thomas Shelite PhD replaced Omar Saldarriaga PhD. He has a post-doc position in our lab.

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Bruno Travi*
Project Role:	*PI*
Researcher Identifier	eRA Commons (NIH) BrunoTravi
Nearest person month worked:	*4*
Contribution to Project:	*Overall scientific supervision and administration of project*

Funding Support:	

Name:	*Alejandro Castellanos-Gonzalez*
Project Role:	*Co-I*
Researcher Identifier	eRA Commons (NIH) ALCASTEL
Nearest person month worked:	*3*
Contribution to Project:	*Participated in lab evaluations of RAP-LF and collaborated in the evaluation of strains from Peru together with NAMRU-6 investigators*
Funding Support:	

Name:	*Thomas Shelite*
Project Role:	*Post-doc*
Researcher Identifier	eRA Commons (NIH) 5NULL9
Nearest person month worked:	*6*
Contribution to Project:	*Participates in lab evaluations of RAP-LF improving the sensitivity of the test using multiple retrospective samples stored at UTMB*
Funding Support:	

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period

Nothing to Report

(**note**: the Partnering PI at NAMRU-6, Andres Lescano PhD was replaced by Robert V. Gerbasi LT USN; this change will be reflected in NAMRU-6 annual report)

Other organizations involved as partners (already reported in year 1)

Organization Name: Fundacion Oswaldo Cruz-FIOCRUZ
Location of Organization: Brazil
Partner's contribution to the project
In-kind support: Species and strains of *Leishmania* isolated from patients in endemic areas of cutaneous leishmaniasis
Facilities: Laboratory facilities of Dr. Renato Porrozzi at FIOCRUZ to carry out *Leishmania* identification using the RPA-LF test.
Collaboration: FIOCRUZ staff (PhD student) collaborated in the evaluation of *Leishmania* strains

Organization Name: Centro Internacional de Entrenamiento e Investigaciones Médicas-CIDEIM

Location of Organization: Colombia
Partner's contribution to the project
In-kind support: Delivery from the lab of Dr. Nancy Gore Saravia of *Leishmania* strains isolated from patients in endemic areas of cutaneous leishmaniasis

Organization Name: Yale School of Public health
Partner's contribution to the project
In-kind support: Delivery of *Leishmania major* strains from the lab of Dr. Diane McMahon-Pratt

Organization Name: Lancaster University
Location of Organization: UK
Partner's contribution to the project
In-kind support: Delivery of *Leishmania major* and *Leishmania enriettii* strains from the lab of Professor Paul Bates